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Characteristics of Membrane-Bound Lectin in Developing *Phaseolus vulgaris* Cotyledons[1]

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ABSTRACT

Cotyledons of developing *Phaseolus vulgaris* L. cv Greensleeves seeds were labeled for 2 to 3 hours with ^{3}H-amino acids, and newly synthesized phytohemagglutinin (PHA) was isolated by affinity chromatography with thyroglobulin-Sepharose. The presence of 1% Tween in the homogenate increased the yield of radioactive PHA by 50 to 100%. Isopycnic sucrose gradients were used to show that this detergent-released PHA was associated with the endoplasmic reticulum (ER), and pulse-chase experiments showed that the half-life of the PHA in the ER was 90 to 120 minutes. Since PHA is transiently associated with the ER and accumulates in protein bodies, we postulate that this rapidly turning over pool of PHA in the ER represents protein en route to the protein bodies. The PHA in the ER has the same sedimentation constant as mature PHA and is capable of agglutinating red blood cells. The observations substantiate earlier claims that plant cells contain membrane-bound lectins. However, they also indicate that these lectins are not necessarily functional components of the membranes with which they are associated, but may represent transport pools of lectin normally localized in other cellular compartments.

The existence of membrane-bound lectin in the organs of higher plants is still a matter of some controversy. Bowles *et al.* (6) reported that Triton X-100 extracts of membranes from leaves, shoots, and roots of soybean possess high hemagglutinating activity. Recently Pueppke *et al.* (12) re-examined the question and concluded that the only organelle fractions containing hemagglutinating activity were from cotyledons of genotypes which also contain non-organelle-bound soybean agglutinin. They found no membrane-associated lectin in other soybean organs. They suggested that the lectin associated with the membranous organelles of cotyledons was not a cytoplasmic contaminant, but cautioned that corroborating evidence is needed to unequivocally establish the existence of membrane-bound lectin.

Seed lectins such as soybean agglutinin, castor bean agglutinin, pea lectin, and PHA[2] have all been shown to occur in the protein bodies of the cotyledons, together with the reserve proteins (1, 3, 10, 17, 18). Reserve proteins are made on membrane-bound polysomes (4, 11) and sequestered in the ER (2, 5, 7) prior to transport to the protein bodies (7). If lectins have a similar site of synthesis (4, 13) and pathway of transport, the ER probably contains a pool of lectin molecules en route to the protein bodies. In this paper, we provide evidence that this is indeed the case for phytohemagglutinin, the major seed lectin of *Phaseolus vulgaris*.

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[2] Abbreviations: PHA, phytohemagglutinin; PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Materials. Seeds of *Phaseolus vulgaris* L. cv Greensleeves (Burpee Seed Co., Riverside, CA) were grown as described (4). Experiments were carried out with cotyledons weighing 200 to 275 mg, when the accumulation of phaseolin and PHA is rapid (4, 15). Organic chemicals were purchased from Sigma Chemical Co. unless otherwise indicated. ^{3}H-Amino acids were purchased from New England Nuclear Co.

Radioactive labeling was carried out with excised cotyledons as described (14). The labeled tissue was collected by cutting a thin slice from the cotyledons with a razor blade. The remainder of the cotyledon was discarded. Homogenization, the use of Sepharose 4B to separate organelles from soluble molecules, and the use of continuous and discontinuous sucrose gradients have all been described (7, 16). NADH-Cyt *c* reductase was assayed as described (4).

Affinity Chromatography. The affinity chromatography procedure of Felsted *et al.* (9) was used to separate PHA from other cellular proteins. Porcine thyroglobulin was linked to cyanogen bromide-activated Sepharose (Pharmacia) according to the specifications of the manufacturer. The thyroglobulin-Sepharose was used as an affinity gel in 0.25-ml portions in small plastic columns. The columns were washed exhaustively with PBS (0.15 M NaCl in 10 mM K-phosphate, pH 7.4), then with PBS containing 1 M NaCl, and then with 2 ml 50 mM glycine-HCl, pH 3.0, containing 0.5 M NaCl. The low pH displaced the PHA from the affinity gel, and the gel could be reused after washing with PBS. The radioactive proteins eluted from the affinity gel were subjected to SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie Brilliant Blue. There were only two polypeptides, and they co-chromatographed with the two polypeptides of authentic PHA (mol wt = 34,000 and 30,000). Fluorography showed that these two polypeptides were labeled and that no other radioactive polypeptides were present on the gel.

Rate Zonal Sucrose Gradients. The ER was treated with 1% Tween and centrifuged at 100,000*g* for 60 min. The supernatant, a portion of the soluble homogenate, and 0.5 mg purified PHA were loaded on linear sucrose gradients (8 to 25% [w/w] sucrose in PBS) and centrifuged at 150,000*g* for 40 h (35,000 rpm in the SW 41 rotor of the Spinco-Beckman ultracentrifuge). Fractions were collected and used for determination of total radioactivity incorporated into protein, radioactivity in PHA, and ability to agglutinate red blood cells.

Agglutination of Red Blood Cells. Blood cells (from M.J.C.) were washed with physiological saline (0.9% NaCl) and resuspended in 10 × the original volume. Aliquots of 0.2 ml were mixed with 0.2 ml test solution and allowed to agglutinate for 1 h. Scoring was done with the unaided eye and the results are

expressed in arbitrary units. Under the conditions used here, 2 μg of PHA produced a positive agglutination reaction.

SDS-Polyacrylamide Gel Electrophoresis. Separation of polypeptides was carried out as described by Bollini and Chrispeels (3), using a ratio of acrylamide to bisacrylamide of 200:1.

RESULTS

Membrane-Associated PHA. To find out if cotyledons contain PHA which is only extractable in the presence of detergent, we labeled bean cotyledons with ^{3}H-amino acids and then homogenized the tissue in PBS with or without 1% Tween. Radioactive PHA was isolated from the homogenate with thyroglobulin-Sepharose and recovered from the affinity gel, and radioactivity was determined (Table I). The results show that the presence of 1% Tween in the homogenizing medium and during the extraction with thyroglobulin-Sepharose increased the amount of newly synthesized PHA which could be extracted by more than 50%.

Next, we fractionated an extract into a soluble and an organelle fraction, and determined the effect of Tween on both fractions. The tissue was homogenized in 12% sucrose, 100 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, and fractionated on a discontinuous sucrose gradient of 16% (w/w) sucrose over 35% (w/w) sucrose in the same buffer into an organelle fraction and a soluble fraction. The conditions of homogenization disrupt the protein bodies so that their contents become part of the soluble fraction of the homogenate. The organelle and soluble fractions were divided in two portions, and PHA was extracted with or without 1% Tween. The results (Table II) show that Tween did not increase the recovery of PHA from the soluble fraction, but increased the recovery of newly synthesized PHA from the organelle fraction by more than 10-fold.

PHA in the ER. To find out which organelles contained the radioactive PHA, the organelle fraction was fractionated on linear isopycnic sucrose gradients. The cotyledons (six cotyledons weighing 250 mg each) were labeled for 2 h with 15 μCi ^{3}H-amino acids each, and the radioactive tissue was homogenized in 12% sucrose and 100 mM Tris-HCl, pH 7.8, containing either 2 mM $MgCl_2$ or 1 mM EDTA. The homogenates were fractionated on Sepharose 4B in the same medium, and the organelles were recovered. The organelles were then fractionated on linear sucrose gradients (16–48% [w/w]) and each gradient fraction was challenged with thyroglobulin-Sepharose in the presence of 1% Tween. Radioactivity in PHA was determined after elution from the affinity gel. The fractions were also assayed for NADH-Cyt *c* reductase, a marker enzyme for the ER. The results (Fig. 1) show that in the presence of EDTA both the PHA and the ER-marker enzyme NADH-Cyt *c* reductase had an average density of 1.14 g cm^{-3}, whereas in the presence of 2 mM $MgCl_2$, the density of both was around 1.18 g cm^{-3}. There was no change in the position of Cyt *c* oxidase (mitochondria) or inosinediphosphatase (dictyosomes) on the gradients (data not shown). These results strongly indicate that newly synthesized PHA is associated with the ER.

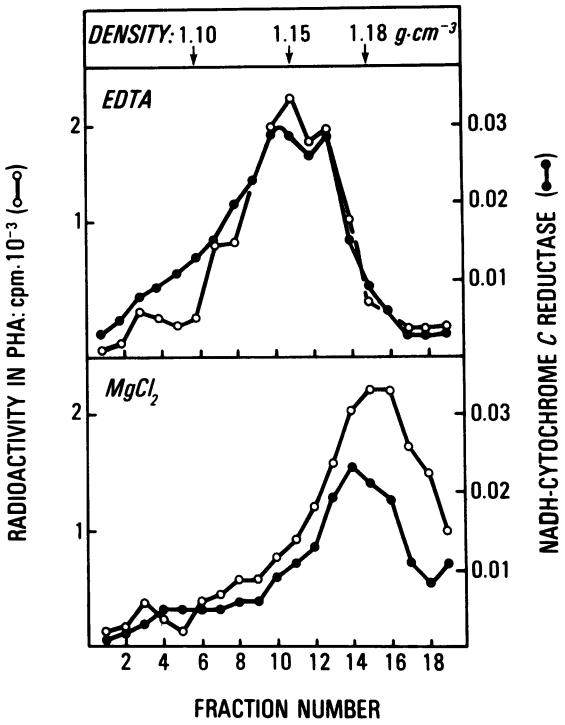

FIG. 1. Distribution of radioactive PHA and NADH-Cyt *c* reductase on sucrose gradients. Details are as in Table II except that the homogenates contained either 1 mM EDTA or 2 mM $MgCl_2$. The organelles were isolated with a Sepharose 4B column and then fractionated on linear 16 to 48% (w/w) sucrose gradients in the same media. The fractions were challenged with thyroglobulin-Sepharose in the presence of 1% Tween, and the radioactivity in the recovered PHA was determined. NADH-Cyt *c* activity is expressed as $\Delta A_{550} \cdot min^{-1}$ cotyledon^1.

To find out whether the newly synthesized PHA remains associated with the ER after its synthesis, we carried out pulse-chase experiments with excised cotyledons. Cotyledons (two per time point; 200 mg each) were labeled with 25 μCi ^{3}H-amino acid each for 1, 2, and 3 h. Some cotyledons were transferred after 3 h to a nutrient medium containing 4% (w/v) sucrose and 10.7 g/L of asparagine, and further incubated (chase) for 1.5, 3, or 8 h. The radioactive tissue was homogenized in 12% (w/w) sucrose in 100 mM Tris-HCl, pH 7.8, with 1 mM EDTA, and the homogenates were fractionated on discontinuous sucrose gradients of 16 over 35% (w/w) sucrose in the same medium. The organelles and load portions were collected and challenged with thyroglobulin-Sepharose in the presence of 1% Tween, and the radioactive PHA was recovered. The data (Fig. 2) show that, at first, newly synthesized PHA was preferentially associated with the organelles (1 h of labeling) and that PHA in the soluble fraction became labeled later on. When the radioactivity was chased, radioactive PHA

Table I. *Effect of Tween on the Extraction of PHA from a Homogenate*

Four cotyledons (250 mg each) were labeled for 3 h with 7.5 μCi ^{3}H-amino acids each. The radioactive tissue was homogenized in PBS containing 0 or 1% Tween. The extracts were challenged with thyroglobulin-Sepharose and the radioactive PHA was recovered

Total Incorporation	Incorporation in PHA
cpm × 10^{-3}/cotyledon	%
347 9.9	2.8
362 16.9	4.7

Table II. *Effect of Tween on the Extractability of PHA from the Organelles and Soluble Fraction of a Homogenate*

Six cotyledons (275 mg each) were labeled for 2 h with 7.5 μCi ^{3}H-amino acids each. The radioactive tissue was homogenized in 100 mM Tris-HCl with 1 mM EDTA and 12% (w/w) sucrose and fractionated on a discontinuous gradient of 16% (w/w) sucrose and 35% (w/w) sucrose in the same medium. The organelles (on top of the 35% sucrose) and the load portion of the gradient were recovered and challenged with thyroglobin-Sepharose in the absence or presence of 1% Tween. The PHA was recovered from the affinity gel, and radioactivity was determined.

Treatment	Soluble Fraction	Organelles
	cpm in PHA/cotyledon	
No Tween	1800	325
1% Tween	2072	4020

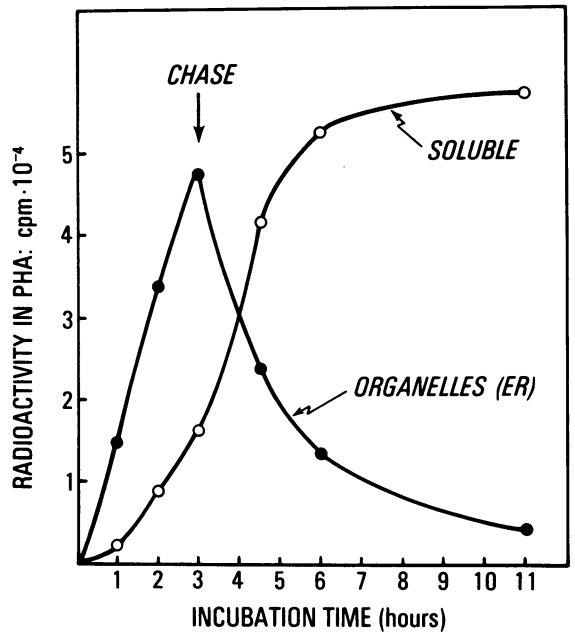

FIG. 2. Radioactivity in PHA in the ER and soluble fraction during pulse-chase labeling of cotyledons. Cotyledons (200 mg; two per time point) were labeled with 25 μCi ^{3}H-amino acids each for up to 3 h, and then rinsed and transferred to a drop of nutrient medium for up to 8 h. The homogenates were fractionated into ER and soluble fractions on discontinuous sucrose gradients, and PHA was isolated with thyroglobulin-Sepharose from each fraction.

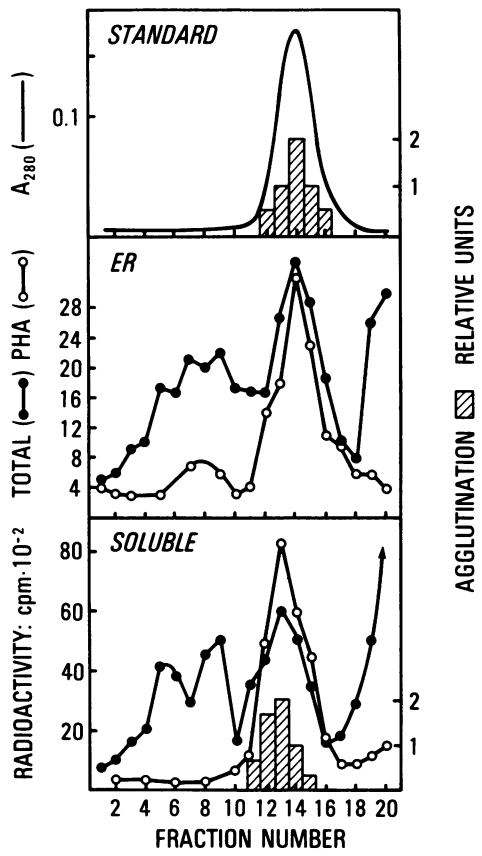

FIG. 3. Rate zonal sucrose gradients of PHA associated with the ER and soluble fractions. Purified PHA (top panel), a detergent extract of ER obtained from cotyledons labeled with 15 μCi ^{3}H-amino acids for 2 h (middle panel), and the soluble portion of a homogenate of cotyledons labeled with ^{3}H-amino acids for 6 h (bottom panel) were loaded on linear sucrose gradients (8–25% [w/w] in PBS) and centrifuged for 40 h at 150,000*g*. The fractions were collected and used to determine *A* at 280 nm (purified PHA), ability to agglutinate red blood cells (purified PHA and soluble portion of cotyledons extract) incorporation of radioactive amino acids into total protein (after precipitation with TCA and collection on membrane filters), and incorporation into PHA (after affinity extraction with thyroglobulin-Sepharose). Radioactivity shown represents incorporation into ER (20% of fraction) and ER-associated PHA (total fraction), or radioactivity incorporated into soluble protein (total fraction) and PHA in soluble protein fraction (total).

disappeared from the ER with a half-life of 90 to 120 min, and continued to accumulate in the soluble portion of the homogenate. Inasmuch as this soluble portion contains the cytosolic proteins as well as the contents of the protein bodies, we assume that the radioactive PHA which leaves the ER is being transported to the protein bodies, as is the case for vicilin and legumin in pea cotyledons (7).

Comparison of the Properties of ER-Associated and Soluble PHA. To find out if the PHA which is associated with the ER has the ability to agglutinate red blood cells, we purified PHA from the ER of developing cotyledons. Cotyledons (16; 275 mg each) were homogenized in 12% (w/w) sucrose containing 100 mM Tris-HCl, pH 7.8, and 1 mM EDTA, and the ER was isolated on a discontinuous gradient of 16% (w/w) sucrose 35% (w/w) sucrose in the same solution. The ER was dissolved with 1% Tween and the PHA was purified by affinity chromatography with thyroglobulin-Sepharose. After elution, the pH was adjusted to 7.0, and the *A* at 280 nm was measured; agglutination tests were performed as described. We found that the PHA obtained from the ER had the same specific activity of agglutination as that obtained from mature seeds. A positive agglutination was obtained with 200 μl of a PHA solution with $A_{280} = 0.010$ which represents 2 μg PHA.

To find out if the PHA associated with the ER was in the 6.4 S form characteristic of mature PHA (3), we determined the sedimentation constants of newly synthesized PHA present in the ER and in the soluble fraction of a homogenate (Fig. 3). Cotyledons were labeled with ^{3}H-amino acids and the homogenate was fractionated into ER and soluble fractions. The detergent-solubilized ER, soluble fraction of the sucrose gradient, and authentic PHA were loaded on separate sucrose gradients and centrifuged for 40 h at 150,000*g*. The gradients were fractionated and each fraction was challenged with thyroglobulin-Sepharose to determine incorporation of radioactivity into PHA and the location of the PHA on the sucrose gradients. Centrifugation for 40 h allowed the purified PHA to sediment ⅔ into the sucrose gradient (top panel, Fig. 3). The newly synthesized PHA present in the ER (middle panel) and in the soluble fraction (bottom panel) was in the same position on the gradient. The ER also contained a small amount of PHA with a lower sedimentation value. This may represent single chains which have not yet been assembled into oligomers. When determining the S value of vicilin in the ER of developing pea cotyledons, we made a similar observation. Most of the newly synthesized vicilin was in the mature 7 to 8 S form, with a smaller amount in 3 to 4 S form (8).

Finally, we compared the mobility of the polypeptides of PHA on SDS-polyacrylamide gels. Two sets of four cotyledons (200 mg each) were labeled with ^{3}H-amino acids for 3 h (for the isolation of ER-associated PHA) or for 4 h followed by a 24-h chase with nutrient medium (for the isolation of soluble PHA). The radioactive tissue was homogenized and the subcellular fractions were isolated as described. The ER was solubilized with 1% Triton X-100 in PBS, and PHA was then isolated from the ER and soluble fraction with thyroglobulin-Sepharose. The isolated PHA and the original ER and soluble fractions were subjected to SDS-polyacrylamide gel electrophoresis and a fluorograph was prepared (Fig. 4). The results show that the affinity column selected

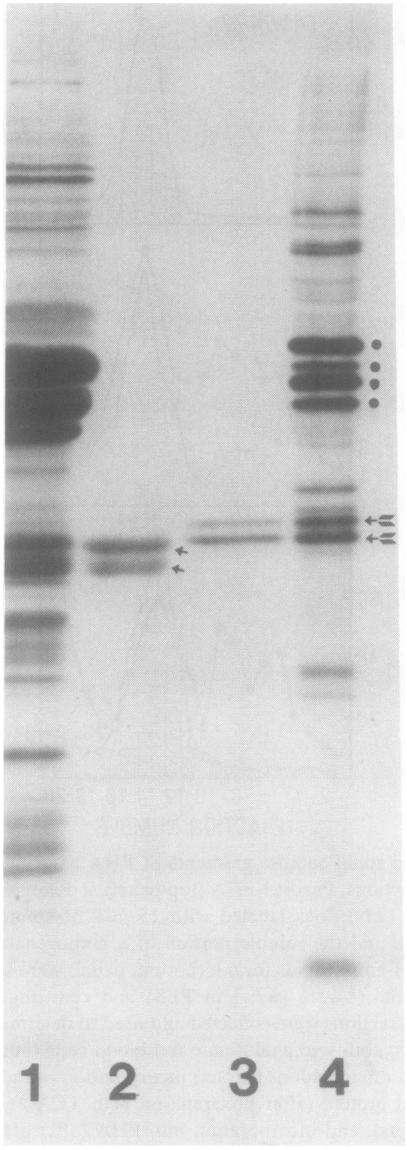

FIG. 4. Fluorograph of newly synthesized PHA fractionated by SDS-polyacylamide gel electrophoresis. Cotyledons were labeled for 3 h (for isolation of ER) or for 4 h followed by a 24-h chase (for isolation of soluble fraction) and the PHA was isolated by affinity chromatography. Lane 1, soluble fraction; lane 2, PHA isolated from soluble fraction; lane 3, PHA isolated from ER; lane 4, ER. Arrows indicate the two polypeptides of PHA, and dots indicate the four polypeptides of phaseolin.

only the two polypeptides of PHA from the many polypeptides which were labeled either in the ER or in the soluble fraction. A comparison of lanes 2 and 3 shows that there is a slight increase in mobility as the polypeptides are transferred from the ER to the soluble fraction (protein bodies). Such an increase in mobility also takes place for the polypeptides of phaseolin(s). The exact nature of this difference in mobility remains to be elucidated.

SUMMARY

The results presented here indicate that the cotyledons of developing seeds contain membrane-bound lectin capable of agglutinating red blood cells. The lectin (PHA) is associated with the ER and constitutes the newly synthesized PHA which is transiently associated with the ER, and is en route to the protein bodies. The PHA in the ER has the same sedimentation constant as PHA isolated from mature seeds. The polypeptides of ER-associated PHA have a slightly lower mobility on SDS-polyacrylamide gels than the polypeptides of mature (protein body) PHA. Any attempt to demonstrate that plant membranes or organelles contain lectins will have to take account of the fact that these lectins may not be 'in residence' in these organelles, but are on their way to other cellular compartments such as protein bodies, or are being secreted.

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